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MUSTARD GAS SURROGATE, 2-CHLOROETHYL ETHYLSULFIDE (2-CEES), INDUCES CENTROSOME AMPLIFICATION AND ANEUPLOIDY IN HUMAN AND MOUSE CELLS

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14. ABSTRACT
Mustard gas is a simple molecule with a deadly past. First used as a chemical weapon in World War I, its simple formulation has raised concerns over its use by terrorist organizations and rogue governments. Mustard gas is a powerful vesicant and alkylating agent and confers painful blisters on epithelial surfaces and increases incidence of cancer. The mechanism of mustard gas toxicity and tumorigenesis is not well understood, but is thought to be mediated by its ability to induce oxidative stress and DNA damage. Centrosomes are small, non-membrane bound organelles that direct the segregation of chromosomes during mitosis through the formation of the bipolar mitotic spindle. Cells with more or less than two centrosomes can segregate their chromosomes unequally, resulting in chromosome instability, a common phenotype of cancer cells. In our studies, we show that subtoxic levels of 2-chloroethyl ethylsulfide (2-CEES), a mustard gas analog, also induce centrosome amplification and chromosome instability in cells, which may hasten the mutation rate necessary for tumorigenesis. These studies offer an explanation why those exposed to mustard gas exhibit higher incidences of cancer than unexposed individuals of the same cohort.

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Mustard gas surrogate, 2-chloroethyl ethylsulfide (2-CEES), induces centrosome amplification and aneuploidy in human and mouse cells

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Abstract

Mustard gas is a simple molecule with a deadly past. First used as a chemical weapon in World War I, its simple formulation has raised concerns over its use by terrorist organizations and rogue governments. Mustard gas is a powerful vesicant and alkylating agent and confers painful blisters on epithelial surfaces and increases incidence of cancer. The mechanism of mustard gas toxicity and tumorigenesis is not well understood, but is thought to be mediated by its ability to induce oxidative stress and DNA damage. Centrosomes are small, non-membrane bound organelles that direct the segregation of chromosomes during mitosis through the formation of the bipolar mitotic spindle. Cells with more or less than two centrosomes can segregate their chromosomes unequally, resulting in chromosome instability, a common phenotype of cancer cells. In our studies, we show that subtoxic levels of 2-chloroethyl ethylsulfide (2-CEES), a mustard gas analog, also induce centrosome amplification and chromosome instability in cells, which may hasten the mutation rate necessary for tumorigenesis. These studies offer an explanation why those exposed to mustard gas exhibit higher incidences of cancer than unexposed individuals of the same cohort.

Introduction

Mustard gas (β, β'-dichloroethyl sulfide) was originally synthesized in 1822 by a French chemist named César-Mansuète Despretz (Despretz, 1822). Since then, several different analogs and derivatives of mustard gas have been developed including the nitrogen and oxygen mustards and half mustard, 2-chloroethy ethylsulfide (2-CEES)(Wang *et al.*, 2012). As for mustard gas, its most documented role in human history include its use as a chemical weapon during World

Wars I and II and the Iran-Iraq war in the 1980s (Duchovic and Vilensky, 2007; Hay, 1993). Most recently, concerns have been raised about countries like Libya and Syria stockpiling mustard gas munitions and whether or not that is a threat to other countries or even their own citizens (Duelfer, 2012; Hough, 2011).

Mustard gas is a vesicant (blistering agent) and is typically encountered exogenously, restricting its acute effects primarily to the skin, lungs, and eye (Aasted *et al.*, 1987; Smith *et al.*, 1995). Its chronic effects include dermal, ocular and respiratory problems as well (Nishimoto *et al.*, 1970; Scholz and Woods, 1947), but neurological (Gadsden-Gray *et al.*, 2012; Thomsen *et al.*, 1998) and reproductive complications (Azizi *et al.*, 1995), and cancers of various organs have also been observed (Doi *et al.*, 2011; Iravani *et al.*, 2007; Zojaji *et al.*, 2009), suggesting that mustard gas can elicit systemic effects. Of interest to our lab is the higher incidence of cancer observed in those that have manufactured mustard gas (Easton *et al.*, 1988; Nishimoto *et al.*, 1983; Takeshima *et al.*, 1994; Yamakido *et al.*, 1996).

Chromosome instability (CIN), also called genetic instability or genomic instability, is a common phenotype of cancers (Pihan *et al.*, 1998). It is characterized by the gain and/or loss of chromosomes, which is thought to hasten the mutation rate necessary for tumorigenesis. There are many mechanisms that are thought to drive chromosome instability, one of which is through centrosome amplification. Centrosomes are small, non-membrane bound organelles that are composed of two microtubule-based centrioles surrounded by a protein matrix called the pericentriolar material. Centrosomes function to organize the microtubule (MT) network and play a central role in organizing the bipolar mitotic spindle. During G_1 of the cell cycle, each daughter cell of the previous mitotic event has only one centrosome. For the next mitotic

event to be successful, the cell must duplicate the single centrosome only once during S phase. As the cell moves into mitosis, the two centrosomes can then form the bipolar mitotic spindle. If a cell duplicates its centrosome more than once, multipolar spindles can form during mitosis and chromosomes can segregate unequally, leading to CIN (Fukasawa, 2005).

Many proteins have been shown to regulate centrosome duplication so that only two centrosomes are present as a cell enters mitosis. Some of these proteins function as targets or mediators of mustard gas toxicity, including p53, poly(ADP-ribose) polymerase (PARP), and NF-κB (Bhat *et al.*, 2000; Fukasawa, 2005; Liu *et al.*, 2011; Ruff and Dillman, 2010; Tong *et al.*, 2007). Additionally, oxidative stress, which is generated by mustard gas (Pal *et al.*, 2009), has been shown to induce centrosome amplification in cells treated with hydrogen peroxide (Chae *et al.*, 2005). Thus, we hypothesized that mustard gas might adversely affect centrosome biology, specifically, centrosome number, which might contribute to the increase in cancer incidences observed in those that manufacture the chemical. Here we show for the first time that 250 µM 2-CEES induces centrosome amplification in both Saos2 and NIH3T3 cells. Additionally, we show that exposure of cells to 250 µM 2-CEES increases chromosome instability as exhibited by an increase in aneuploidy in treated cells.

Methods and Materials

Cell Culture

Saos2 (human osteosarcoma) and NIH3T3 (murine embryonic fibroblasts) cells were obtained from ATCC (HTB-85 and CRL-1658, respectively) and cultured in complete media: Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml

penicillin and 100 µg/ml streptomycin (Hyclone) for maintenance and all experiments. Cells were incubated in an environment of 10% CO_2 and 37°C in a humidified incubator for all experiments.

Experimental Design

For all experiments, 80-90% confluent plates were washed with PBS and then trypsinized with TrypLE Express (Life Technologies) followed by gentle agitation to dislodge attached cells. Dislodged cells were then harvested in a volume of complete DMEM necessary to produce a hemacytometer count of 200-300 live cells via trypan blue assay (See below). Cells were then plated in dishes at a density that would result in about 85% confluency after overnight incubation. Cells were then incubated in 2-CEES for 24 hours, after which a specific assay was performed.

Trypan Blue Assay

Cells were plated in 6-well plates and treated with 2-CEES as previously mentioned. After 2-CEES treatment, each well was trypsinized with 50 µl of TrypLE Express (Life Technologies) and harvested with 350 µl of complete DMEM. 50 µl of the 400 µl cell suspension was then diluted 1:1 with trypan blue and the number of live cells was immediately counted on four large squares (1 mm^2 each) of a hemacytometer. The total live cell density was then determined by the following equation.

$$\frac{Live\ cell\ count\ in\ 4\ large\ squares}{.0004} \times 2\ (dilution\ factor) = cells/ml$$

Resazurin Assay

Cells were plated in 24-well plates and treated with 2-CEES as previously mentioned. Additionally, a well containing media only was prepared for each 2-CEES concentration to act as a blank. After 2-CEES treatment, 4.4 mM resazurin (Sigma) was prepared fresh in water and diluted 1:100 in each well (experimental and blank wells) followed by a two hour incubation. Fluorescence levels in each well were measured at 550 nm (excitation) and 600 nm (emission) for 1.2 seconds using a CareStream Image Station 4000MMPRO imaging system. A 51 × 51 pixel region of interest (ROI) was generated over the center of each well and the mean intensity of each well was measured. Final fluorescence values were determined by subtracting the blank value of each 2-CEES concentration from the same well containing cells ($600\ nm_{cells} - 600\ nm_{blank}$) and normalized to 0 μM 2-CEES treated wells.

Immunofluorescence

Cells were grown and treated on glass coverslips. After 2-CEES treatment, cells were washed in PBS (135 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.2) and fixed in 4% formalin/methanol (Fisher) for 20 minutes at room temperature followed by permeabilization with 1% Nonidet P-40 (Fisher) in PBS for 10 minutes at room temperature. Cells were blocked in 15% NGS (Life Technologies) for 1 hour and then gently washed by dipping coverslips into three separate beakers of PBS. Cells were then incubated in rabbit-anti-γ tubulin antibody (Cell Signaling) diluted in PBS for 45 minutes at room temperature. Cells were then washed with PBS for 15 minutes on a rocker and then exposed to AlexaFluor 594 conjugated goat-anti-rabbit IgG

antibody (Life Technologies) diluted in PBS for 45 minutes at room temperature in the dark. Cells were washed in TBS (150 mM NaCl, 20 mM Tris, pH 7.4) for 15 minutes on a rocker in the dark followed by nuclear counterstaining with 500 nM 4',6-Diamidino-2-phenylindole (DAPI, Sigma) for 10 minutes in the dark at room temperature. Lastly, coverslips were washed again in TBS for 10 minutes on a rocker in the dark at room temperature and then mounted on coverslips using Fluoromount-G (Southern Biotech).

Metaphase Spread and Fluorescence* In Situ *Hybridization

Saos2 and NIH3T3 cells were plated in 6 cm dishes and treated with 2-CEES as described above. After 2-CEES treatment, media in both treated and untreated dishes was replaced with normal, complete media without 2-CEES and incubated for an additional 5 days. Cells were split as necessary to maintain subconfluent cultures. After the 5 day incubation, cells were treated with 0.5 µg/ml colcemid (Gibco) for 4 hours. Cell media was harvested and retained since it contains many mitotic cells. The remaining adherent cells were washed with PBS, which was retained with the media, and the cells were trypsinized and harvested. The media, wash, and cells were combined and centrifuged to pellet cells. The pellet was resuspended in warm (37°C) hypotonic buffer (75 mM KCl). 5 drops of fixative solution (methanol:glacial acetic acid, 3:1) was added while gently vortexing. Cells were centrifuged, resuspended in fixative solution, and incubated for 10 minutes at room temperature 3-4 times with cells suspended in a final volume of 300 µl of fixative solution. Positively-charged glass slides (Fisher) were soaked in water for 1 minute, followed by a 2 minute soak in methanol. Slides were dipped in water until the water ran smooth. Fixed cells were then dropped onto a prepared, positively-charged glass slide

(Fisher) from a height of 18-24 inches and then allowed to air dry. Cells were then stained with 500 nM DAPI in the dark at room temperature for 10 minutes, followed by 3-4 quick washes with TBS. A coverslip was added to each slide using Fluoromount-G (Southern Biotech). Images of mitotic cells were collected using a Zeiss AxioImager A2. A minimum of 50 cells was collected for each treatment condition, and the number of chromosomes per cell was counted and grouped in ranges of 5 chromosomes (Figure 3).

Results

Effect of 2-CEES on cell viability

We first needed to determine an effective concentration of 2-CEES to use in our experiments. If the concentration was too high, cells might simply die before a measurement could be made. If the concentration was too low, an effect on centrosome number might not be observable. To determine an appropriate concentration, we established a toxicity curve (Figure 1). We first determined an appropriate concentration range to test by doing a literature search (Cook and Van Buskirk, 1997; Qui *et al.*, 2006; Rancourt *et al.*, 2012; Tewari-Singh *et al.*, 2011). The results indicated a range of 0-1,000 µM treated anywhere from 2 hours to 24 hours. Ultimately, we decided to evaluate a range of 0-1,000 µM over a 24 hour period.

To do this, cells were plated overnight and exposed to 2-CEES in increasing concentrations for 24 hours. Cell viability was then evaluated using both trypan blue and resazurin assays. Trypan blue is a blue dye that is taken up by dead cells, but actively excluded from live cells (Altman *et al.*, 1993). Thus, dead cells appear blue, while live cells appear colorless in a microscope. In this

case, the total number of live cells was determined using a hemacytometer (Figure 1A). As expected, the concentration of live cells decreased as the concentration of 2-CEES increased. To corroborate our findings with the trypan blue assay, we also employed a fluorescent assay where resazurin, a blue, non-fluorescent molecule is reduced to the pink, fluorescent resorufin by a myriad of suspected enzymes, mostly mitochondrial (O'Brien *et al.*, 2000). The resulting chemical change can then be monitored either colorimetrically by measuring the absorbance at 600 nm or fluorometrically at peaks of 560 nm (excitation) and 590 nm (emission), which is directly proportional to the number of live cells in each well (Sigma product insert). Here, cells were seeded and treated as with the trypan blue assay, but after 2-CEES treatment, cells were incubated in 44 µM resazurin for 2 hours followed by fluorescence detection at 550 nm (excitation) and 600 nm (emission). The mean fluorescence intensity of each well was recorded, blank values subtracted for each treatment, and normalized to 0 µM 2-CEES (Figure 1B). Saos2 and NIH3T3 cells showed an overall decrease in live cells as the concentration of 2-CEES increased up to 1000 µM in both trypan blue and resazurin assays. Taking together published treatment concentrations and our empirical data, we decided to treat cells with 250 µM 2-CEES over a 24 hour period for our subsequent studies.

2-CEES induces centrosome amplification

Since mustard gas is known to induce oxidative stress (Pal *et al.*, 2009), and since oxidative stress has been shown to induce centrosome amplification (Chae *et al.*, 2005), we next wanted to investigate the possibility that 2-CEES could induce centrosome amplification in Saos2 and NIH3T3 cells. To do this, we again treated cells with 2-CEES for 24 hours and then

immunostained for γ-tubulin and counterstained with 4',6-Diamidino-2-phenylindole (DAPI) to detect nuclei. Centrosomes appeared as a perinuclear, intensely fluorescent dots in interphase cells (Figure 3A) or monopolar, bipolar, or multipolar spindles in mitotic cells (Figure 3B). After immunostaining, the number of centrosomes in at least 100 cells was counted. Each cell was categorized as having one (1), two (2), or more than two (>2) centrosomes per cell, and the percent of cells in each category was then calculated and graphed (Figure 2A and B). As shown, most untreated cells exhibited one or two centrosomes per cell, with a small percentage showing more than two, which is likely due to the fact that these cells lack the tumor suppressor protein p53 (Fukasawa *et al.*, 1996). However, cells treated with 2-CEES show a significant increase in the number of cells exhibiting centrosome amplification in both Saos2 and NIH3T3 cells, indicating that 2-CEES can induce centrosome amplification in these cell types.

We also evaluated the effect of different concentrations of 2-CEES on centrosome amplification and found a concentration effect in that as the amount of 2-CEES increased from 0 to 500 μM, the amount of centrosome amplification increased as well (Figure 2C).

2-CEES-induced centrosome amplification correlates with increase aneuploidy

Since chromosome instability is a characteristic of many cancers, it is possible that mustard gas might hasten the development of cancer through centrosome amplification and subsequent chromosome instability. To determine if 2-CEES treatment can induce chromosome instability, we performed a metaphase spread to determine the number of chromosomes in cells after 2-CEES treatment. To this end, Saos2 and NIH3T3 cells were treated for 24 hours with 2-CEES and

then incubated in 2-CEES-free media for an additional five days. This allowed cells with multiple centrosomes to go through multiple rounds of mitosis and thereby increase the degree of anticipated aneuploidy in treated cells. After five days in 2-CEES-free media, a metaphase spread was prepared and chromosomes were stained with DAPI (Figure 3B). The number of chromosomes was counted in at least 50 cells in each condition, and the results were grouped into increasing groups of 5 chromosomes (Figure 3A). Saos2 cells are human osteosarcoma cells and are thus expected to have around 46 chromosomes, while the modal chromosome number for NIH3T3 cells is unknown and may be highly varied, although our data suggests that it may be polyploid at around 70-80 chromosomes per cell. Our data shows an increase in the number of cells with extremely low or high number of chromosomes in cells treated with 2-CEES and a concomitant decrease in the number of cells in the normal, modal range of chromosomes per cell compared to untreated cells (Figure 3A). This is more apparent in NIH3T3 cells than Saos2, which is likely due to the fact that Saos2 cells are slower growing than NIH3T3. Regardless, our data indicates that 2-CEES induces aneuploidy in Saos2 and NIH3T3 cells.

Discussion

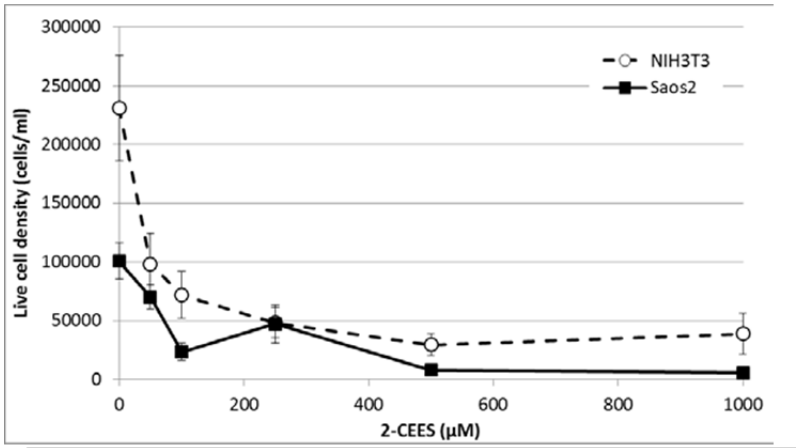
Here we document the first report of the mustard gas analog, 2-CEES, inducing centrosome amplification in human and mouse cells. We show that subtoxic levels of 2-CEES increase the number of cells with more than 2 centrosomes per cell compared to untreated cells. Furthermore, an increase in chromosome instability, as indicated by an increase in aneuploidy in 2-CEES-treated versus untreated cells, correlates with centrosome amplification as well.

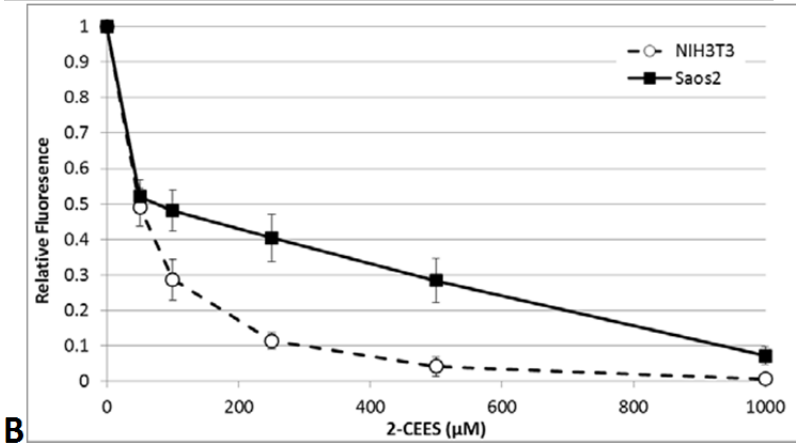
Respiratory epithelia, ocular tissue, and skin are the acute, primary targets of mustard gas toxicity (Nishimoto *et al.*, 1970; Scholz and Woods, 1947), because they are the first tissue exposed. However, sequelae in deeper tissues have also been shown to correlate with mustard gas exposure, including neurological (Gadsden-Gray *et al.*, 2012; Thomsen *et al.*, 1998), reproductive (Azizi *et al.*, 1995), cardiac (Gholamrezanezhad *et al.*, 2007), thyroid (Zojaji *et al.*, 2009), gastrointestinal (Iravani *et al.*, 2007), and possibly others, suggesting that mustard gas may be able to affect deeper tissues either directly or indirectly by generating systemic increases in reactive oxygen species (ROS) or DNA damage. The direct effects of mustard gas on deeper tissues in animal models are not available, most likely due to the difficulty in uncoupling its direct effects from those that are due to its ability to generate ROS. Regardless, our studies show that both embryonic fibroblasts (NIH3T3) as well as human osteosarcoma cells (Saos2) exhibit biological changes when exposed directly to 2-CEES, indicating that mustard gas toxicity is not cell type specific.

The mechanism driving centrosome amplification in cells exposed to 2-CEES, and potentially mustard gas, is obviously unknown. However, some proteins that have been shown to be involved in centrosome duplication have also been shown to be targets of mustard gas or mediate mustard gas toxicity. For example, poly(ADP-ribose) polymerase-1 (PARP-1) has been shown to localize to the centrosome and its loss results in centrosome amplification in mouse embryonic fibroblasts (Kanai *et al.*, 2003; Wang *et al.*, 2007). Likewise, mustard gas has been shown to cause PARP cleavage in normal human epidermal keratinocytes (Rosenthal *et al.*, 2000; Stoppler *et al.*, 1998), indicating that loss of PARP due to mustard gas-induced cleavage may result in the centrosome amplification we observed.

p53 also plays a role in centrosome amplification and mustard gas toxicity. p53 is a tumor suppressor protein that is absent or nonfunctional in the vast majority of cancers (Vogelstein *et al.*, 2000). It has been shown to regulate centrosome duplication through its transcriptional activity (Fukasawa, 2005) and by its localization to the centrosome (Shinmura *et al.*, 2007). *In vitro*, cells treated with mustard gas or 2-CEES show increased expression (Inturi *et al.*, 2011; Jowsey *et al.*, 2009) and activation by phosphorylation of p53 (Everley and Dillman, 2010). The same is seen *in vivo* (Dillman *et al.*, 2005; Sharma *et al.*, 2009) in rats and mice, respectively. These results are most likely due to activation of the p53-mediated DNA damage repair pathway, which results in increased and stable expression of p53 (Jowsey *et al.*, 2009, 2012). Epidemiologically, there is a correlation between p53 mutations and lung cancer in individuals exposed to mustard gas both as victims of attacks (Hosseini-khalili *et al.*, 2009) and as workers in mustard gas factories (Takeshima *et al.*, 1994). However, it cannot be determined if the mutations existed prior to mustard-gas exposure, or if the mustard gas exposure induced the mutations that may have led to the increased lung cancer incidence observed in these individuals. These data suggest that initial, acute exposure to mustard gas initiates stress pathways, in part mediated by p53, while long-term, or higher dose, exposures cause DNA damage, in particular the p53 gene, that leads to tumorigenesis. In our experiments, 250 μM 2-CEES induced centrosome amplification and chromosome instability in Saos2 and NIH3T3 cells, which lack functional p53, thus supporting the idea that p53 loss is a precursor to mustard gas-induced carcinogenesis. We are currently studying the role of p53 in mustard gas-induced centrosome amplification and chromosome instability.

Figures A





B

Figure 1. Toxicity curves of 2-CEES on Saos2 and NIH3T3 cells. NIH3T3 (dotted line) and Saos2 (solid line) cells were incubated in 2-CEES for 24 hours and then subjected to trypan blue assay (A) or resazurin assay (B). For trypan blue assay, cells were harvested and diluted 1:1 in trypan blue and the number of clear cells was counted using a hemacytometer. For resazurin assay, cells were incubated in 44 µM resazurin for 2 hours and then fluorescence was measured at 550 nm (excitation) and 600 nm (emission) for 1.2 seconds and normalized to 0 µM 2-CEES. Results are the average of at least three independent experiments. Error bars represent standard error.

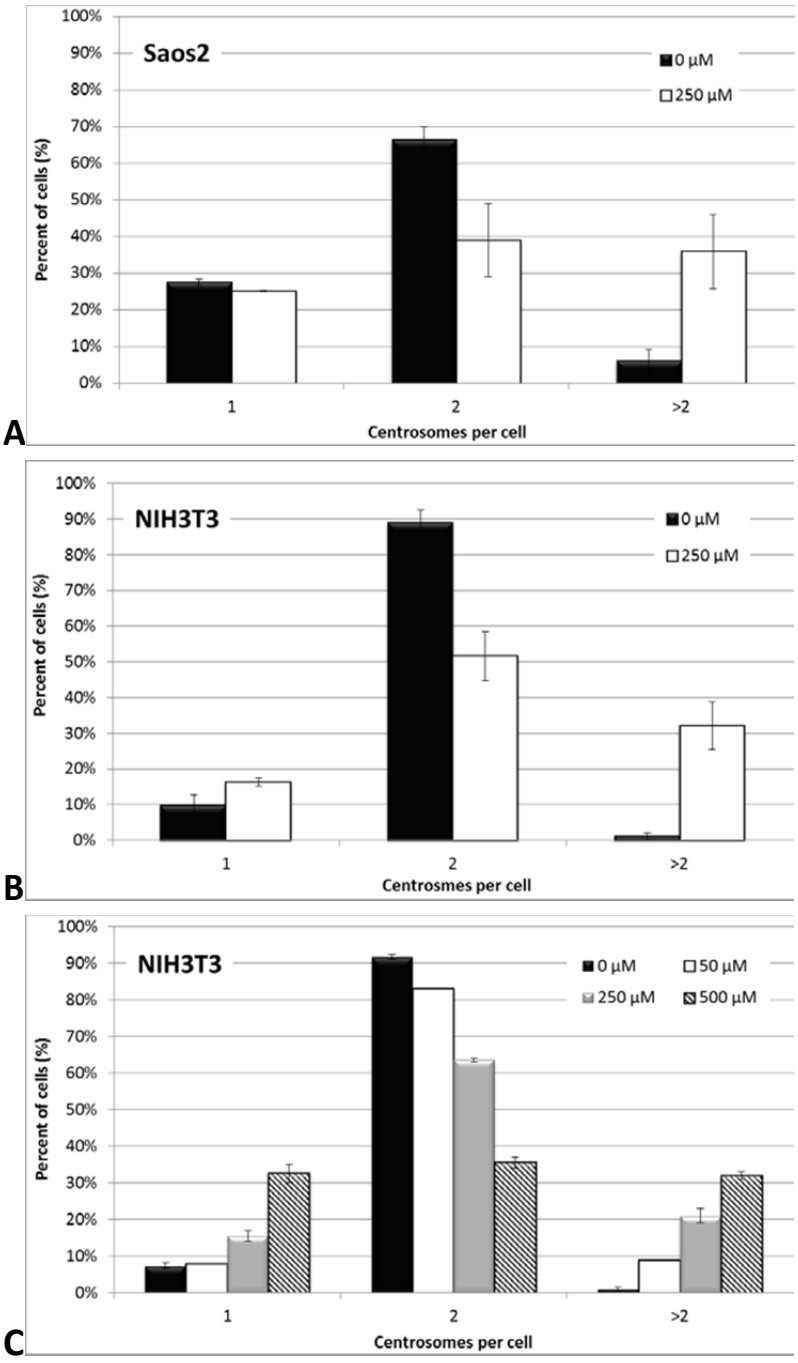


Figure 2. 2-CEES induces centrosome amplification. Saos2 (A) and NIH3T3 (B) cells were untreated or treated with 0 or 250 µM (A and B) or 0, 50, 250, or 500 µM (C) 2-CEES for 24 hours and then immunostained using antibodies against γ-tubulin and counterstained with DAPI. The number of centrosomes was counted in each of at least 100 cells. $p < 0.05$ comparing treated to untreated cells with more than 2 centrosomes per cell, except for 50 µM, which was only performed one time.

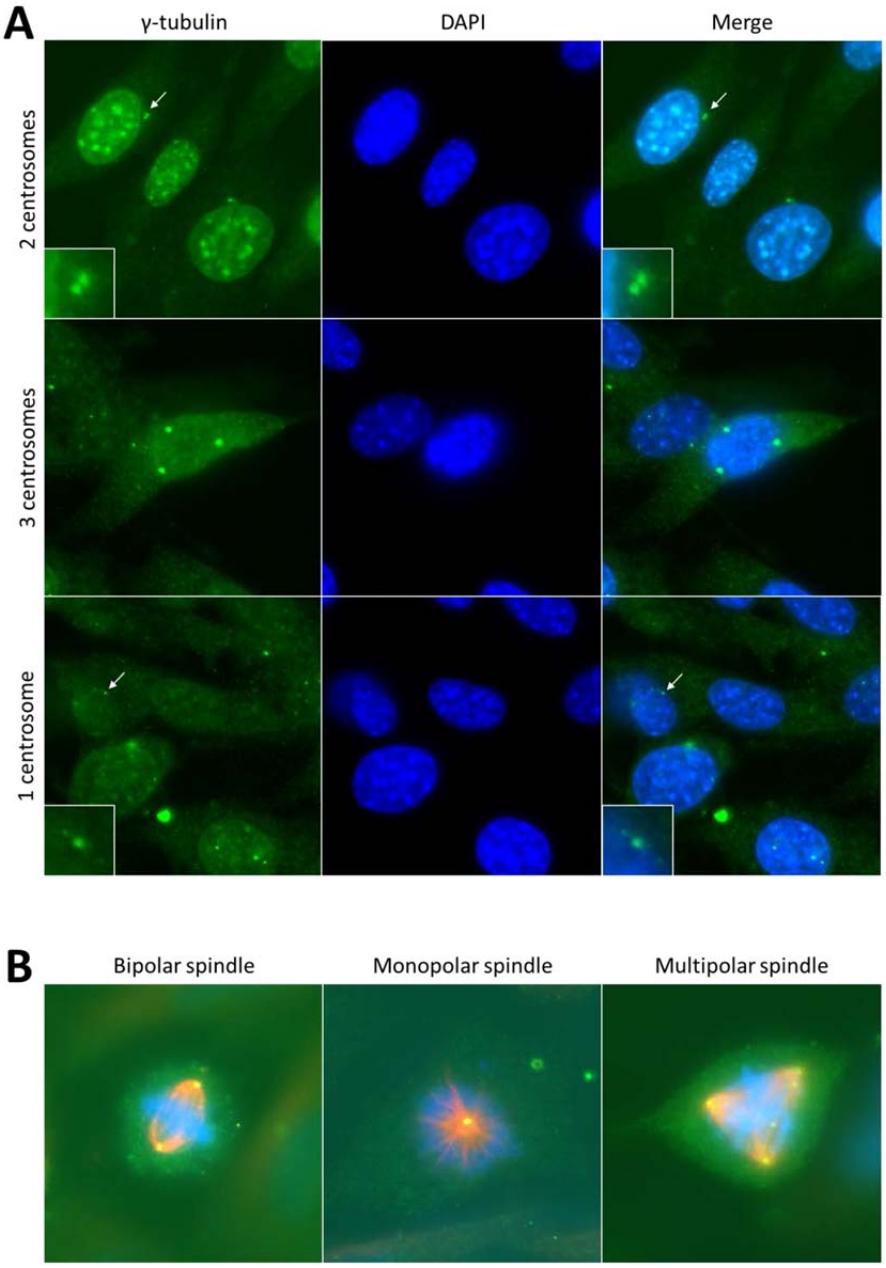


Figure 3. 2-CEES-induced centrosome amplification results in monopolar and multipolar mitotic spindles. Sample images of NIH3T3 cells treated with 2-CEES for 24 hours and then immunostained with γ-tubulin to indicate centrosomes (green) and DAPI to indicate DNA (blue) (A), or γ-tubulin (green), DAPI (blue), and α-tubulin (red) to indicate microtubules (B).

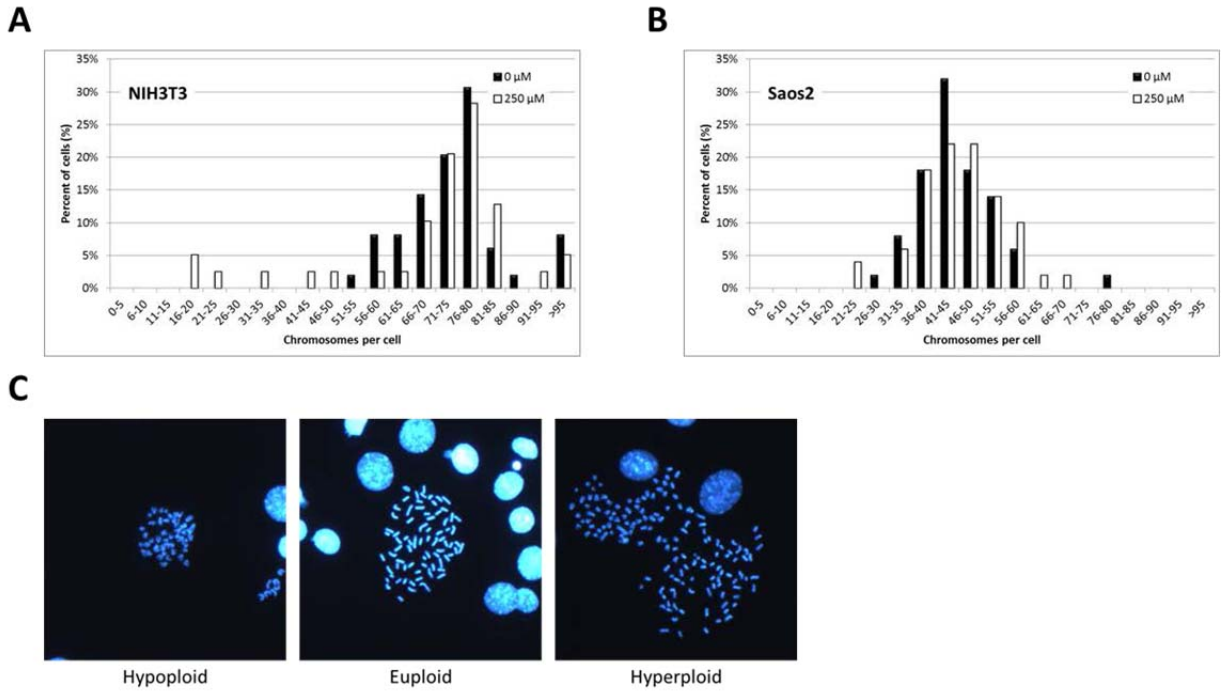


Figure 4. 2-CEES induces chromosome instability. NIH3T3 (A) and Saos2 (B) cells were treated with 0 or 250 µM 2-CEES for 24 hours and then incubated in normal media for 5 days. Cells were treated with 0.5 µg/ml colcemid, fixed, stained with DAPI to visualize chromosomes (C).

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